

Microbiological Process Report

Natural and Induced Fluorescence in Microscopic Organisms

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The fluorescence of microscopic organisms may be either natural or induced. Organisms which do not have sufficient intrinsic fluorescence can be impregnated with specific chemicals called fluorochromes or fluors. Both types of fluorescence depend upon the presence of materials which emit radiation at a wavelength different from that of the light source. These substances absorb radiant energy and during the return to their original energy state fluorescence occurs. In applying this phenomenon to microscopic studies of biological specimens, ultraviolet light is generally used and cells glow brilliantly against a dark background. It is of interest that many of the great inherent capabilities of the ultraviolet microscope as known today were recognized 60 years ago by Köhler and von Rohr (1904) when they designed and built the first of these instruments.

AUTO-FLUORESCENCE

Many bacteria, actinomycetes, algae, and fungi fluoresce naturally in ultraviolet light; and the presence of invading organisms, especially actinomycetes, has been detected clinically, as well as in plants and animals, by this method. The invasion of potato by *Streptomyces scabies*, for example, has been quickly determined by its auto-fluorescence (Richards, 1943). In a survey of more than 50 cultures of actinomycetes (Krassil'nikov and Kalakutskii, 1959) all were found to fluoresce under polarized light when grown on agar; however, the mycelium of these same cultures grown in liquid medium on shakers was not fluorescent. Although changes in auto-fluorescence with age have been found in algae (Wassink and Katz, 1939) as well as fungi (Rudaya, 1958), the toxicity of *Fusarium sporotrichiella* has been correlated with the degree of fluorescence of the strain (Rubinstein, 1956) and the intensity of auto-fluorescence on agar has also been recommended for use in the screening of cultures for the production of riboflavin (Dikanskaya, 1953) and the antibiotic oxytetracycline (Rudaya, 1958). The possible taxonomic classification of various groups of actinomycetes by their differing auto-fluorescence has been suggested (Krassil'nikov and Bekhtereva, 1956). The differentiation of tumor tissue by this technique has also been investigated

(Herley, 1944), and fluorescence studies on chloroplast development and chlorophyll formation have also been undertaken (Epstein, De La Tour, and Schiff, 1960; Krasnovsky and Bystrova, 1960).

Although the fluorescence of the pseudomonad bacteria has been known for many years, only recently have any attempts been made at identification of the fluorescent material (Elliot, 1958). The pigments of the fluorescent azotobacter are also only now under chemical investigation (Johnstone, Pfeffer, and Blanchard, 1959), although their fluorescence had been suggested earlier as a means of species identification (Johnstone and Fishbein, 1956); and the pigments elaborated by certain aspergilli, which were once thought to be riboflavin, have characteristics which indicate they are probably pteridine derivatives (Wolf, 1957) somewhat similar to those of the azotobacter. The fluorescing pigments of some actinomycetes have also been studied (Cortese, 1930, 1931) as well as those of certain higher fungi (Dhéré and Castelli, 1937; Josserand and Nétien, 1938, 1939). Much work remains to be done before the phenomenon of auto-fluorescence in microscopic organisms is understood.

Although Schrotter had observed the absorption of ultraviolet light by bacterial spores as early as 1906 and Smakula and Laser in 1934 had described ultraviolet absorption maxima in a variety of tissues, it remained for Caspersson in 1936 to recognize the significance of these phenomena and to demonstrate that the intense ultraviolet absorption of cells was probably due to their nucleic acids and proteins. Caspersson (1950) and his colleagues have subsequently developed this technique into an attack on the whole problem of the localization and metabolism of nucleoproteins within the cell. The ultraviolet absorption of living nuclei during growth and division has been intensively studied (Ris and Mirsky, 1949; Walker and Yates, 1952). Since in cytological structures protein concentrations range between 10 and 40% and nucleic acids between 1 and 8% (Nurnberger, 1955), favorable conditions for this approach exist. However, the contributions to cytological cellular techniques with naturally occurring ultraviolet micro-absorption appear, with our present knowledge, to be somewhat restricted in their scope, when compared

either with simple and selective staining procedures or with the combination of cell fractionation and microchemical analysis.

INDUCED FLUORESCENCE

In contrast, induced fluorescence in the long wave ultraviolet range appears to offer many opportunities for successful application in the biological field. In addition to its cytological aspects, this technique has been used for the visualization of small objects, for particle counting, for specific reactions, and for the localization and identification of chemicals and organisms. Of paramount importance is the fact that the circulation, concentration, and excretion of fluorescent or fluorescence-tagged materials may now be studied within living organisms.

The fluorescent compounds per se whose distribution has been followed in human or animal tissues include the antimalarial atabrine (Jailer, 1945), aromatic diamidines and hydroxystilbamidine (Snapper et al., 1951), an anti-pernicious anemia factor (Jacobson and Simpson, 1946), riboflavin (Bodine and Fitzgerald, 1946), and vitamin A (Popper, 1941). The mode of action and localization of fluorescent antibiotics has also been studied by this technique (Milch, Rall, and Tobie, 1957; Newton, 1955), and fluorescence has become an integral part of the chromatography of steroids (Haines and Drake, 1950) as well as of antibiotics (Regna and Solomons, 1950).

In studies with fluorescence-tagged materials, fluors or fluorochromes are employed. These may be standard, colored dyes, which visibly stain the specimen and which, on irradiation, fluoresce with a similar or dissimilar color; other fluors remain colorless until irradiated. Some of the uses of fluors include labeling for localization of antigen in tissue cells (Coons and Kaplan, 1950; Coons et al., 1942); labeling for bacterial and viral infiltration (Hagemann, 1937; Maximovick and Mitchenko, 1959; Rapp, Gordon, and Baker, 1960); discrimination of certain types of cancer cells (Bertalanffy, 1960; Bertalanffy, Masin, and Masin, 1956, 1958; Friedman, 1950); identification of specific bacterial genera (Kaufman and Weaver, 1960); studies on bone structure and development (Ribelin, Masri, and Deeds, 1960); staining techniques for tubercle bacilli (Hagemann, 1937; Kuper and May, 1960), trypanosomes (Jansco, 1932; Strugger, 1948a), and malarial organisms (Fishl and Singer, 1935); bacterial counts (Greene and Hesseltine, 1950; Strugger, 1948b); localization of inhaled dust (Yogoda and Donahue, 1946); and detection of antibiotics in milk (Hargrove, Lehman and Matthews, 1958).

The nature, color, and applications of many fluors are listed by Richards (1955). Dilute aqueous solutions of 0.1% concentration are generally satisfactory; and the staining period ranges from a few seconds to over-

night, depending on the material, the fluor, and the concentration. Berberine sulfate and fluorescein are probably the most generally used fluors; the acridines are also extensively employed. By combining several fluors or by using them successively, it is possible to obtain differential coloring in the tissues. Many fluors combine selectively with certain cellular components. Some will fluoresce only within a narrow range of acidity, and buffers may be needed to preserve the color or to maintain the tissue in proper condition. In addition to pH, the fluorescence of fluors is dependent upon temperature, viscosity, electrolyte concentration, and concentration of the fluorescent material itself. Gurr (1951) has summarized many of the procedures for impregnating biological materials with fluorochromes.

The apparent brightness of the fluorescence of the fluor at a given magnification depends on the intensity of the light source, the efficiency with which it is concentrated on the specimen by the illuminating system of the microscope, the cytological location and efficiency of the fluor, the amount of the emitted fluorescence collected by the microscope objective, and losses from absorption within the microscope.

The light emission from a fluorescent particle itself is dependent on the size and shape of the particle and the difference in refractive index between the particle and its environment. Emitted rays which have an incidence more oblique than the critical angle for the interface between the particle and its surroundings cannot leave the particle. Although inorganic materials can be broken into sizes and shapes that will circumvent total internal reflection, biological materials cannot be changed in this manner.

Although the use of fluors to visualize specimens was initiated in 1914 by Provazek, there was an extremely slow development of this technique due largely to the lack of easily usable ultraviolet sources. In the 1930's German workers investigated the use of fluorescent fluors as a means of labeling bacteria, viruses, malaria organisms, and trypanosomes (Fishl and Singer, 1935; Hagemann, 1937; Jansco, 1932). At the same time vital fluors were also investigated. Some of the first applications of these vital fluors were in living animals, and in 1932 Singer studied kidney tissue of the frog, rat, and mouse subsequent to the injection of fluorescein. Pick and Zukerkandl (1935) also investigated this technique. Other fluors, including acriflavine, primulin, rhodamine B, and thioflavin S, were employed by Keller (1949) with submerged animal tissues. Keller and Pisha (1947) stained living plants and animals with rhodamine B after fluorescein thus permitting the appearance of two fluorescent colors in their preparations. Strugger (1940, 1947), using the fluor acridine orange on dead and living bacterial and plant cells, observed some differences in their fluorescent pattern.

Although all of the acridine compounds possess the property of fluorescence, the diamino acridines are the most active in this respect. Since maximal activation of the fluorescence of these substances occurs in the blue-violet region of the spectrum, quartz optical components are not required; a high pressure mercury vapor lamp provides an adequate light source and with a dark-field condenser and suitable filtration, the fluorescent images stand out clearly against a black background. Excellent discussions of ultraviolet microscopy have been published by Blout (1953), Nurnberger (1955), and Richards (1955).

The diamino acridine basic fluors, including acriflavine, acridine orange, and acridine yellow have a pronounced affinity for cellular nucleoproteins and nucleic acids (De Bruyn, Robertson, and Farr, 1950). When administered *in vivo* they react with nuclear deoxyribonucleic acid (DNA) and nuclear ribonucleic acid (RNA) and display little affinity for extra nuclear proteins (Bertalanffy and Bickis, 1956; De Bruyn et al., 1953). After tissue fixation these fluors do, however, have an affinity for all nucleoproteins regardless of their localization within the cell (De Bruyn et al., 1953). With acridine orange there are specific fluorescences for RNA and DNA (Armstrong, 1956; Armstrong and Niven, 1957); structures containing DNA, such as nuclear chromatin, show a greenish-yellow fluorescence, whereas RNA-containing material gives a bright flame-red color. Cellular age has also been reported to influence fluorescence with acridine orange as well as other fluors (Naumova, 1960).

One of the most important controlling factors in the fluorescence of the diamino acridines is the pH of the staining solution. The most striking differential fluorescence has been obtained within a pH range of 1.5 to 3.5 for acridine orange and 3.5 to 5.0 for acridine orange R, the 5-phenyl derivative (Armstrong and Niven, 1957; Niven, 1959). A pH of 7.0 has, however, been employed for studies *in vivo* (Morthland, De Bruyn, and Smith, 1954). The detection of the diamino acridines in animal tissues following subcutaneous injection has shown (De Bruyn et al., 1950) that 3,6-diamino-10-methyl acridinium chloride remained in the tissues for the longest time; its presence could be detected for 12 days. Acridine orange could be seen for only 30 hr and acridine yellow for 20 hr. The interaction of the diamino acridines with nucleoproteins has formed the basis of techniques used for observations on virus-infected cells (Armstrong and Niven, 1957; Armstrong and Hopper, 1959), on the multiplication of bacteriophage in lysogenic cells (Anderson, 1957), and on the multiplication of yeast cells (Freifelder and Uretz, 1960).

Paralleling the use of the diamino acridines in studies *in vivo*, investigations were continued with fluorescein. The early work of the Germans was extended into the field of antigens by Coons et al. in 1942. Their research

demonstrated that a fluorescent compound could be conjugated with antibody molecules without loss of activity. Heidelberger, Kendall, and Soo Hoo in 1933 had introduced visible labels into the study of immunological reactions, and the following year Marrack (1934) reported that an antibody molecule could be coupled with a dry molecule without losing its capacity for specific reaction with the antigen. Subsequent research has established an effective labeling technique for the localization of antigen in tissue cells (Coons, 1958, 1959a, b, c, Coons and Kaplan, 1950; Coons, Leduc, and Conolly, 1955; Coons et al., 1950; Marshall, Eveland, and Smith, 1958; Tobie, 1958). The fluorescent fluorochrome, fluorescein, is chemically linked to protein molecules of the antibody and the fluorescent antibody is used as a histochemical reagent. When this labeled antibody is brought in contact with a tissue section containing specific antigen, the antibody molecules react with the antigen; the unreacted antibodies and other labeled proteins can be washed away; and the fluorescent label can be observed under the fluorescence microscope. Research workers have successfully traced bacterial polysaccharides and foreign animal proteins when injected into mice, and they have located viral and rickettsial antigens in infected cells. Antibodies labeled with fluorescein have come into widespread use for the localization of various kinds of antigenic molecules in tissues. The staining of bacteria with fluorescent antibody by Moody, Goldman, and Thomason (1956) has become increasingly important; this technique may hasten the diagnosing of many diseases, and it is reported (Moody et al., 1959) to be, in general, more sensitive than agglutination.

During the past few years several papers applying the fluorescent antibody technique have appeared. These publications pertain to such diverse studies as the attachment of chemicals to animal tissues in the production of allergies (Chase, Slizys, and Dukes, 1959; Goldstein, Slizys, and Chase, 1960); the estimation of infectious virus units in tissue culture (Rapp, Seligman, and Gordon, 1959); the measurement of polyoma virus synthesis in tumor cells (Sachs and Fogel, 1960); the chloramphenicol-induced accumulation of fluorescent substance in bacteria (Wolfe and Hahn, 1960); the evaluation of connective tissue disease (Kaplan, 1959); the uptake of proteins by animal tissues (Holter and Holtzer, 1959); the histological study of infectious canine hepatitis (Coffin, Coons, and Cabasso, 1953); and the diagnosis of rabies (Goldwasser and Kissling, 1958), poliomyelitis (Kalter, Hatch, and Ajello, 1959), Colorado tick fever (Burgdorfer and Lackman, 1960), typhoid and paratyphoid (Thomason, Cherry, and Edwards, 1959; Truant, 1959), pertussis (Kendrick, Eldering, and Eveland, 1960), cholera (Finkelstein and La Brec, 1959), brucellosis (Biegeleisen and Moody, 1960; Kaufman and Cherry, 1960), erysipelas (Marshall

et al., 1959), and infections involving: *Escherichia coli* (Stulberg, Cohen, and Page, 1960; Thomason and Cherry, 1960; Thomason, Cherry, and Ewing, 1959), *Corynebacterium* (Jones and Moody, 1960), *Candida* (Kemp and Solotorovsky, 1960), *Cryptococcus* (Kase and Marshall, 1960), *Endamoeba* (Goldman, 1954), *Gonococcus* (Deacon et al., 1960), *Listeria* (Smith, Marshall, and Eveland, 1959), *Mimae* (Eveland et al., 1959), *Haemophilus influenzae* (Page, Caldrony, and Stulberg, 1960), *Staphylococcus aureus* (Pittman and Moody, 1960), *Streptococcus* (Moody, Ellis, and Updyke, 1958; Redys, Ross, and Borman, 1960), *Malleomyces* (Moody et al., 1956; Thomason, Moody, and Goldman, 1956), and the microaerophilic actinomycetes (Slack and Moore, 1960). With the synthesizing of fluorescein isothiocyanate (Riggs, 1957; Riggs et al., 1958), the fluorescent antibody technique has been substantially improved by greater stability and brilliance.

Conjugates of proteins with other fluorochromes have also been investigated. These include β -anthryl isocyanate (Creech and Jones, 1941), nuclear fast red (Clayton, 1954), rhodamine B-isocyanate (Silverstein, 1957), and rhodamine B (Laurence, 1952). None of these compounds has been widely used, either because of difficulty in repeating conjugations or because the products compared unfavorably with fluorescein conjugates. The use of Lissamine Rhodamine B 200 (RB 200) as a fluorescent protein tracer has, however, recently been recommended (Chadwick, McEntegart, and Nairn, 1958). This compound has a brilliant red fluorescence in aqueous solution; in the form of its sulfonyl chloride it can be easily combined with serum proteins, without denaturation, to yield stable conjugates which have a brilliant orange fluorescence. Other sulfonic acid derivatives have also been investigated. Stable fluorescent conjugates of ovalbumin and bovine serum albumin have been prepared with 1-dimethylaminonaphthalene-5-sulfonyl chloride (Weber, 1952a, b). The same compound has been used for the localization of embryonic antigens (Clayton, 1954). The absorption of sulfonic acid derivatives of 1- and 2-naphthylamine with 1-, 2-, or 3-sulfonic acid groupings on bovine serum albumin has been studied by fluorescent microscopic techniques (Laurence, 1952). The fluorescent compound 1-dimethylaminonaphthalene-5-sulfonamido polymyxin, which is comparable in biological activity to polymyxin, has been used to investigate the site of its antibiotic action (Newton, 1955). A fluorescent dichloro-triazinyl dye has been employed (Hess and Pearse, 1959) for the labeling of antibodies, the tracing of antigens (ovalbumin, serum albumin, and gamma globulin), and the demonstration of antigen-antibody reactions in tissue sections.

It is apparent that induced fluorescence is of substantial value to the microbiologist and has become an integral part of the biologist's armamentarium. It is only during the last decade, however, that it has been

applied to any great extent to the investigation of microbiological problems. Applications of induced fluorescence other than those mentioned will undoubtedly be made as the field is further explored and the techniques expanded and improved.

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